ROLE OF 4-CHLOROINDOLE-3-ACETIC ACID IN *VIGNA RADIATA* TREATED WITH COPPER

DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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Dedicated

To

My Parents

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Certificate

This is to certify that the dissertation entitled, **"Role of 4-chloroindole-3-acetic acid in *Vigna radiata* treated with copper"** submitted for the degree of Master of Philosophy in Botany is a bonafied research work carried out under my guidance and supervision at the Aligarh Muslim University, Aligarh, India by **Miss Shazia Khanam** and that no part of it has been submitted for any other degree or diploma.

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Shazia Khanam

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Chapter - 1

Introduction

INTRODUCTION

Soil contamination with heavy metals is becoming a major problem all over the world, leading to the loss in agricultural productivity and hazardous health effects However, their availability to the plants is determined by the natural processes, especially on the lithogenic and pedogenic ones and also an anthrogenic factors (Keversen *et al.*, 1998). The heavy metals that have a direct impact on the plants include Fe, Zn, Cu, Mn, Co, Ni, Cd, Pb and Cr. Out of them copper is an essential micronutrient for plant growth and development but in excess it is toxic for plants. It is a redox-active metal that functions as an enzyme activator and acts an important part of prosthetic groups of many enzymes. Plant requires approximately 5-30 mg Cu kg^{-1} dry weight (DW) for normal growth (Kabata-Pendias and Pendias, 1992). Copper deficiency usually occurs when Cu concentration is smaller than 5 mg kg^{-1} DW (Marschner, 1995). Excess Cu affects a wide range of biochemical and physiological processes including the inhibition of cell elongation, vegetative growth and reproductive progress, oxidative phosphorylations and protein trafficking (Balsberg Pahlsson, 1989; Fernandes and Henriques, 1991; Ouzounidou *et al.*, 1995; Alaoui Sosse *et al.*, 2004; Brun *et al.*, 2003). It cause malformation of the roots, leaf decoloration and necrosis; reduce total nitrogen content (Van Assche and Clijsters, 1990; Marschner, 1995; Punz and Sieghardt, 1993; Llorens *et al.*, 2000). Moreover. Cu exposure results in increase in free amino acids like proline (Mazen, 2004) and inhibit the activity of nitrate reductase, pigment synthesis, protein metabolism, membrane integrity mineral uptake, chlorophyll content and photosynthesis (Luna *et al.*, 1997; Nielsen *et al.*, 2003; Demirevska-Kepova *et al.*, 2004). Among all the above mentioned processes photosynthesis is most severely affected process.

Although copper is an essential micronutrient for photosynthetic organisms but at higher concentration it is an effective inhibitor of photosynthesis in higher plants (Maksymiec and Baszymski, 1998; Ouzounidou *et al.*, 1998; Varda Caspi *et al.*, 1999). Excess Cu has a toxic effect on the primary reactions of photosynthesis and electron transport in maize (Ouzounidou *et* al., 1997). The primarily target of the toxicity is reaction center of photosystem II (PS II). PS II is more susceptible to copper toxicity than photosystem I (PS I) (Yruela *et al.*, 1996; Ouzounidou *et al.*, 1997; Patsikka *et al.*, 1998; Vinit Dunand *et al.*, 2002). Moreover, excessive Cu induces leaf chlorosis, breakdown of pigments and membrane lipids in barley leaves (Caspi *et al.*, 1999), bean (Shainberg *et al.*, 2001), *Rumex dentatus* (Liu *et al.*, 2004). Excessive copper can catalyze the generation of harmful reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$), which can damage biological molecules (DNA, RNA and proteins) and membranes by inducing lipid peroxidation (Mediouni *et al.*, 2006, Halliwell and Gutteridge, 1984), *Phaseolus vulgaris* (Weckx and Clijsters, 1996). In addition, copper as a transition metal, can particularly catalyze the formation of harmful free radicals (Drazkiewicz *et al.*, 2003). Plants have evolved protective enzymatic and non-enzymatic mechanisms to remove ROS and reduce their deleterious effects. The protective enzymes in plant include catalase (CAT, EC 1.11.1.6), superoxide dismutase (SOD, EC 1.15.1.1), peroxidases (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) etc. while several small molecules such as glutathione (GSH), ascorbic acid (AsA), and carotenoids (Car) provide non-enzymatic protection of the physiological metabolisms (Alscher *et al.*, 1997).

The exogenous application of plant hormones has been found to counter toxic effect of various stresses. It is well recognized that indole-3-acetic acid (IAA) is the major and ubiquitous auxin in plants, which plays a key role in the regulation of plant growth and development (Davies, 1995; Taiz and Zeiger, 2002). The other naturally occurring auxins include 4-Cl-IAA (Engvild, 1985, 1994, 1995; Hatano *et al.*, 1987), indole-3-butyric acid (IBA) and phenyl-acetic acid (PAA) (Normanly *et al.*, 1995; Normanly, 1997; Davies, 2004). However, others are reported to occur in restricted plant groups such as 4-Cl-IAA has been reported in plant mainly belonging to family Fabaceae (Reinecke, 1999; Ali *et al.*, 2006) and in the seeds of *Pinus sylvestris* (Ernstsen and Sandberg, 1986). The strong auxin like activity of this chlorosubstituted auxin has been demonstrated in a number of bioassays (Engvild, 1994; Reinecke, 1999; Ali *et al.*, 2006). In plants, the auxins are characterized principally by their capacity to stimulate cell elongation, in excised stems and coleoptile (Hopkins, 1995). However, they also influence several other developmental responses including cell growth, vascular differentiation, root initiation, development of axillary buds, flowers and fruits, abscission and ethylene production (Ahmad *et al.*, 1987; Hopkins, 1995; Davies, 1995, 2004). It favour seed germination (Ahmad *et al.*, 2001a) and photosynthesis (Ahmad *et al.*, 2001b) with regard to their activity 4-Cl-IAA has been found to possess the strongest auxin like activity in a number of bioassays, whereas in certain cases it has been reported to be 50 fold more active than the commonly occurring auxin (IAA) (Antolic Salopek *et al.*, 1998; Reinecke, 1999), 10 fold more active for elongation of the Avena coleoptile, 100-fold for hypocotyls swellings of the mungbean, and more than 100-fold for root inhibition of Chinese cabbage (Marumo *et al.*, 1974; Bottger *et al.*, 1978). Out of various chloroindole auxins tested seeds germination gave

maximum response to 4-Cl-IAA (Ahmad *et al.*, 2001a) possibly by mediating the effect on hydrolases (Hirasawa, 1989; Ahmad *et al.*, 2001a). The level of nitrate reductase increased in response to 4-Cl-IAA in pea seedlings (Ahmad and Hayat, 1999), mustard plants (Ahmad *et al.*, 2001b) and eggplant seedlings (Hayat *et al.*, 2005), 4-Cl-IAA also enhanced the activity of carbonic anhydrase, chlorophyll content, the rate of photosynthesis and seed yield in *Brassica juncea* (Ahmad *et al.*, 2001b).

The present study was carried out with an aim to explore the ameliorative role of 4-Cl-IAA against heavy copper stress and to work out some remedial measures to overcome the toxicity of copper in plants.

Chapter - 2

Review of Literature

REVIEW OF LITERATURE

Heavy metals make a significant contribution to environmental pollution as a result of anthropogenic activities such as mining, smelting, electroplating, energy and fuel production, power transmission, intensive agriculture, sludge dumping and military operations (Nedelkoska and Doran, 2000). They pose a risk for primary and secondary consumers and ultimately disturb the homeostasis (Zeller and Feller, 1999).

Copper (Cu) is an essential micronutrient for growth and development of plants. Like most micronutrients, Cu is needed in small amounts by the plants. At cellular level Cu plays an essential role in signalling of transcription, protein trafficking, oxidative phosphorylation and iron mobilization (Yruela, 2005). Moreover, Cu is also required in biological systems as a structural component and as cofactor in catalytic activity of enzyme. However, at higher concentrations in soil it can generate causing physiological responses that can inhibit plant growth (Ouazounidon, 1994; Monni *et al.*, 2001), enzyme activity, DNA alteration, protein oxidation, photosynthesis, pigment synthesis, nitrogen and protein metabolism, membrane integrity, and mineral uptake (Luna *et al.*, 1994; Alaoui-Sosse *et al.*, 2004). Cu is also a redoxactive transition metal, but excess concentrations may induce a significant toxic effect by altering the protein function and the enzyme activity (Marschner, 1995). Toxicity may result from the binding of metals to sulfhydryl groups in the protein, leading to the inhibition of activity or disruption of the structure (Meharg, 1994).

The growth of the cell and that of whole plant is drastically reduced by copper toxicity (Ouzounidou *et al.*, 1995; Maksymiec *et al.*, 1995; Alaouo-Sasse, 2004). The presence of excess amount of copper in the soil decreased

the growth of *Phaseolus vulgaris* (Cook *et al.*, 1997), *Brassicaa pekinensis* (Xiong *et al.*, 2006), maize (Tanyolac *et al.*, 2007), *Rumex japonicus* (Ke *et al.*, 2007). Thus at whole plant level high of concentration, Cu can be extremely toxic causing symptoms such as chlorosis and necrosis, stunting of growth, leaf discoloration and inhibition of root growth (Van Assche and Clijsters, 1990; Marschner, 1995). An increase in the concentration of metal in the soil decreased the fresh mass and dry mass of cucumber (Burzynski and Klobus, 2004), *Brassica pekinensis* (Xiong *et al.*, 2006), *Amaranthus tricolour* (Shi-Sheng, 2007). Cell elongation is a complex process depending on cell turgor pressure, synthesis of wall components, as well as on growth regulator contents. There are two major requirements for cell elongation: an increase in cell wall extensibility and solute accumulation to create an internal osmotic potential (Marschner, 1995). Excess copper commonly inhibited cell elongation in *Agrostis tenuis* (Wainwright *and* Woolhouse, 1977), *Zea mays* (Ouzounidou *et al.*, 1995). Excess Cu concentration may similarly inhibit the nodulation in leguminous crop. A high level of copper decreased chlorophyll content and induced alterations in chloroplast structure and composition of thylakoid membrane (Lidon and Henriques, 1991, 1993), *Triticum durum* (Ciscato *et al.*, 1997), wheat (Patsikka *et al.*, 1998, Quartacci *et al.*, 2000). In particular, elevated level of Cu disturbed stacking of grana and stroma lamellae, increase in the number and size of plastoglobuli, and also led to appearance of intrathylakoidal inclusions. Copper can also substitute for Mg in the chlorophyll present in both antenna complexes and photosynthetic reaction centers (Kupper *et al.*, 1996). This may result from chlorophyll degradation and inhibiton of chlorophyll synthesis due to Cu-induced degradation of enzyme δ– Amino Levulinic acid dehydrates (Fernandes and Henriques, 1991). Also

decline in chlorophyll content could result from an indirect effect on N assimilation of the plants; since biosynthesis of chlorophyll is dependent on supply of N. Moreover, elevated level of copper reduced leaf chlorophyll content in *Phaseolus vulgaris* (Zengin and Munzuroglu, 2005), *Brassica pekinensis* (Xiong *et al.*, 2006), sand *Brassica juncea* (Fariduddin *et al.*, 2009).

Photosynthesis is one of the most severely affected process induced by elevated level of heavy metal in the soil. Cu is toxic at high concentrations for photosynthetic organisms (Clijsters and Van Asche, 1985; Maksymiec, 1997). The presence of copper above the threshold limit decreased photosynthesis, stomatal conductance and internal CO_2 concentration in cucumber (Burzynski and Klobus, 2004), photosynthetic activity in rice (Lidon *et al.*, 1993), growth in runner plant (Maksymiec *et al.*, 1995) and the maximum quantum yield of PS II photochemistry assessed by chlorophyll fluorescence in bean plant (Maksymiec *et al.*, 1999). Extensive *in vitro* studies have shown that photosystem II (PS II) is more susceptible to copper toxicity than photosystem I (PS I) (Droppa and Horvath, 1990; Ouzounidou *et al.*, 1997). Both the acceptor and donor side have been proposed as copper inhibitory sites (Bernal *et al.*, 2004; Yruela, 2005). Excess Cu damages the Q_B Binding site resulting in reduction of quantum yield of PS II (Maksymiec *et al.*, 1994, Patsikka *et al.*, 1998). In addition to this it have suggested that the electron flow from tyrosine (Tyr_z) to P_{680} is blocked by toxic concentration of Cu(II) (Yruela, 2005) It was proposed that Cu(II) interacts not only with Try_z, but also with Tyr_D on D2 protein of photosystem (Kralova *et al.* 1994; Sersen *et al.* 1997; Jegerschold *et al.*, 1995, 1999, Sersen *et al.*, 1997). Excess Cu in the plants tissue significantly changed the nitrogen metabolism with a reduction of total nitrogen in *Vitis vinifera* (Llorens *et al.*, 2000) and inhibition of nitrate reductase activity in

Triticum aestivum (Luna *et al.*, 1997), *Brassica pekinensis* (Xiong *et al.*, 2006) and ammonium assimilation in rice (Chen and Kao, 1998), barley (Demirevska-Kepova *et al.*, 2004).

Proline is a proteinogenic amino acid, accepted as an indicator of environmental stress. It has been reported that under heavy metal stress, proline is a commonly accumulated as free amino in plant tissue and serve as a tolerance mechanism in plants (Bassi and Sharma, 1993a, b; Schat *et al.*, 1997). Proline accumulation in plant tissues has been suggested to result from (a) a decrease in proline degradation, (b) an increase in proline biosynthesis, (c) a decrease in protein synthesis or proline utilization, and (d) hydrolysis of proteins (Charest and Phan, 1990). In rice, proline generated under heavy metal stress could be involved in metal chelation in cytoplasm (Roy *et al.*, 1992). An increase in the constitutive proline level have been observed in a copper tolerant genotype of *Armenia meritima* (Farago, 1979). A significant increase in the proline content was observed in the leaves of *Phaseolus vulgaris* (Zengin and Munzuroglu, 2005) and sunflower (Zengin and Kirbag, 2007) on being exposed to copper stress.

The presence of heavy metal in the soil can induce the antioxidative activities in plants (van Assche and Clijsters, 1990; Schutzendubel and Polle, 2002 and subsequently increase the level of antioxidant enzymes due to production of highly toxic oxygen free radicals. It was observed that excess Cu in plants led to oxidative stress inducing changes in the activity and content of some components of the antioxidative pathways i.e., ascorbate peroxidase (APx), monodehydroascorbate reductase (MDHAR), dehydroasrobtate reductase (DHAR), glutathione reductase (GR), guiacol peroxidase in *Silene*

cucubalus (De Vos *et al.*, 1992), leaves (Luna *et al.*, 1994), wheat (Navari-Izzo *et al.*, 1998), *Phaseolus vulgaris* (Gupta *et al.*, 1999), *Arabidopsis thaliana* (Drazkiewicz *et al.*, 2003). It has been reported that Cu increases the activity of peroxidase (POX) in *Phaseolus vulgaris* (Wackx and Clijsters, 1996) *Lemna minor* (Teisseire and Guy, 2000), *Salix viminalis* (Landberg and Greger, 2002), *Rumex dentalus* (Liu *et al.*, 2004), maize (Tanyolac *et al.*, 2007). Similarly enhanced activity of superpxide dismutase (SOD) under Cu stress was observed in *Triticum aestivum* (Luna *et al.*, 1994), *Arabidopsis thaliana* (Alscher *et al.*, 1997), wheat (Navan-Izzo *et al.*, 1998) soybean (Chongpraditnum *et al.*, 1992), *Amaranthus tricolor* (Shi-Sheng, 2007) and in maize (Tanyolac *et al.*, 2007). The ascorbate-glutathione cycle has been reported to be involved in response to excess Cu in *Phaseolus vulgaris* and *Arabidopsis thaliana* (Gupta *et al.*, 1999; Drazkiewize *et al.*, 2003). Devi and Prasad (2005) demonstrated that accumulation of copper led to more active lipid peroxidation, depletion of glutathione (GSH) pools and also altered activities of antioxidant enzyme in *Brassica juncea.* Copper stress increased lipid peroxidation and H_2O_2 content in *Amaranthus tricolor* and wheat (Zhang *et al.*, 2007; (Shi-Sheng, 2007).

Peroxidases (POX) can transform peroxides into non-reactive species. The destruction of H_2O_2 has been shown to be an important function of peroxidases that use ascorbate as the hydrogen donor (Asada, 1994). However, increase in activity of POX is thought to be a common response and can protect plants from various stresses (Chaoui *et al.*, 2004). Peroxidases activities are detected in the majority of cellular constituents, notably in the cytoplasm membrane, and in cell wall where they are ionically or covalently bound (Chaoui *et al.*, 2004).

It is well recognized that indole-3-acetic acid (IAA) is the major and ubiquitous auxin in plants which plays a key role in the regulation of plant growth and development (Davies, 1995). The other naturally occurring auxins included indole-3-butyric acid, phenyl acetic acid and 4-chloroindole-3-acetic acid (Normanly *et al.*, 1995; Normanly, 1997). 4-chloroindole-3-acetic acid (4-Cl-IAA) has been identified in the extracts of vicieae and also its methyl ester in the immature seeds of pea (Marumo *et al.*, 1986; Engvild *et al.*, 1980) and the shoot, root and the cotyledons of etiolated seedlings of pea (Schneider *et al.*, 1985). Similarly, it is also reported in *Pinus sylvestris* (Ernstsen and Sandberg, 1986) and the young leaves of *Vicia faba* (Pless *et al.*, 1984). Out of various chloroauxins (4-Cl-IAA) occurs in plants belonging to the family Fabaceae (Engvild *et al.*, 1978; Katayama *et al.*, 1987) and it has also been also found in the pine seeds (Ernstsen and Sandberg, 1986).

In plants, the auxins are characterized principally by their capacity to stimulate cell elongation in excised stem and coleoptile section (Hopkins, 1995). However, they also influence several other developmental processes in plants, including root initiation, vascular differentiation, development of axilliary buds, flower and fruits (Davies, 1995). The biological activity of 4-Cl-IAA has been tested in a number of bioassays, where it is 1.3 to 50 time more active than IAA (Antolic *et al.*, 1999; Engvild, 1985). 4-Cl-IAA is highly active in Avena coleoptile curvature assay (Gerhold and Muir, 1989). Various studies showed that auxins regulate growth, nitrogen fixation, yield and various enzyme activities. Both IAA and 4-Cl-IAA are required to coordinate the vegetative and reproductive growth of pea plants (Mangus *et al.*, 1997). IAA and 2,4-D have also been found to increase the internode elongation in IAA deficient pea mutatnts (McKay *et al.*, 1994). IAA, IBA are responsible for

rhizogenesis in various plant species (Muller, 2000; Wang *et al.*, 2003). Moreover, 4-Cl-IAA also induced lateral root initiation and their subsequent growth in pea cuttings (Ahmad *et al.*, 1987) and wheat and cucumber seedlings (Stenlid and Engvild, 1987). 4-Cl-IAA is the strongest auxin showing more activity than IAA in terms of growth promotion (Engvild, 1985). High biological activity of 4-Cl-IAA compared to IAA has been demonstrated in a number of biological assays which include wheat and cucumber root inhibition, tomato epinasty, tomato parthenocarpy, mung bean growth inhibition and root initiation and pea pericarp growth (Reinecke *et al.*, 1995). However, wheat roots were inhibited more by IAA than 4-Cl-IAA (Stenlid and Engvild, 1987). 4-Cl-IAA stimulated pea internode section growth, root initiation and ethylene evolution in shoot cuttings, and pericarp elongation (Katekar and Geissler, 1982; Ahmad *et al.*, 1987; Reinecke *et al.*, 1995). 4-Cl-IAA also plays an important role during early and mid seed development in *Vicia faba* (Pless *et al.*, 1984) and may also act as a sink signal (Pless *et al.*, 1984). Exogenous application of 4-Cl-IAA to maize activated growth over and above that of IAA (Fischer *et al.*, 1992; Karcz and Burdach, 1995, 2002; Karcz *et al.*, 1999).

Out of various chloroindole auxins tested, seed germination gave maximum response to 4-Cl-IAA (Ahmad *et al.*, 2001a) possibly by mediating the effect on hydrolases (Hirasawa, 1989; Ahmad *et al.*, 2001a). 4-Cl-IAA also enhanced the activity of carbonic anhydrase (Ahmad *et al.*, 2001b), chlorophyll content, the rate of photosynthesis in *Brassica juncea* (Ahmad *et al.*, 2001a) and *Solanum melongena* seedlings (Hayat *et al.*, 2006). Plants treated with auxins photosynthesized at higher rate and senescence was delayed (Menon and Srivastava, 1984).

Auxin or their substituents have also been reported to stimulate the activity of NR in pea seedlings (Ahmad and Hayat, 1999), mustard plants (Ahmad *et al.*, 2001a) and germinating seeds of chickpea (Ali *et al.*, 2007a). Nitrate reductase (NR) activity in root nodules of *Vigna radiata* increased significantly by the application of GA_3, IAA, salicylic acid in combination with exogenous nitrate (Sekhon *et al.*, 1991). However, IAA in combination with glucose had an antagonistic effect on NR activity in the presence or absence of exogenous nitrate (Sekhon *et al.*, 1991). It is well established that the auxin (IAA or 4-Cl-IAA) increase the uptake of NO_3 (Ahmad and Hayat, 1999) and also affects the transcription/translation process (Woodward and Bartel, 2005). Furthermore, the activity of NR is very much unsteady and is reported to be stimulated in presence of hormones (Knypl and Krystyna, 1979), auxin or its substitutes (Ahmad and Hayat, 1999, Ahmad *et al.*, 1988, 1994) and/or monochloroindole acetic acid (Ahmad *et al.*, 2001b). The order of response to various hormones was 4-Cl-IAA > 7-Cl-IAA > 6-Cl-IAA = 5-Cl-IAA = 4,6-Cl_2-IAA = 4,7-Cl_2IAA > control (Hayat *et al.*, 2006)..In addition of this, the efficiency of the nodules to fix atmospheric nitrogen is also an important process in legumes. Since auxin and cytokinin act as a "Trigger" for cell division in initiation of root nodule (Syno *et al.*, 1976). IAA metabolism plays an important role in early stages of development of symbiotic relations between a leguminous plant and an appropriate symbiotic nitrogen fixing bacterium (Fedorova *et al.*, 2000; Benjamin *et al.*, 2007). IAA at a lower concentration significantly increased plant biomass, nodule number and their mass and nitrogen, phosphorus, potassium contents in *Leucaena leucocephala*, whereas higher doses resulted in steady decline (Hussain *et al.*, 1988). Application of IAA increased nodule number, nodule dry mass, plant dry mass, nitrogenase

activity and total shoot nitrogen in *Cajanus cajan* (Raghuvanshi *et al.*, 1992). A concentration of 300 μM of IAA significantly enhanced the nodulation and delayed nodule senescence whereas, the concentration above 300 μM hastened nodule senescence and also enhanced leghemoglobin content in cowpea (Raghava *et al.*, 1996).

The increase in growth of plant mediated by the auxins involves at least two events (i) the acidification of the cell wall adjoining the plasma membrane and (ii) the activation of genes/protein synthesis (Davies, 2004). In the former case, the auxin causes the extrusion of H^+ ions into the cell wall by activating the membrane bound ATPase thereby acidifying the cell wall. The decrease in pH in the cell wall optimize the conditions for hydrolytic enzymes such as expansions, which break the glycosidic linkage between the carbohydrates units of the cell wall. Simultaneously, the auxins increase the water uptake, relative water content (Ali *et al.*, 2007; Hopkins, 1995) generating a hydrostatic pressure on the cell whereby the cell expands (Hopkins, 1995).

It is an established fact that phytohormones have in inherent role in derepressing specific genes to activate protein synthesis which is expressed by increase in the activity of peroxidase in the seeds treated with 4-Cl-IAA (Moore, 1989). Auxin inducible genes and cDNA with significant sequence similarities to these, have subsequently been identified in other plant species, including *Arabidopsis thaliana* (Abel *et al.*, 1994; Conner *et al.*, 1990; Gil *et al.*, 1994), *Pisum sativum* (Oeller *et al.*, 1993) and *Vigna radiata* (Yamamoto *et al.*, 1992). However, littie information is available on the effects of indole-3-acetic acid (IAA) on plants grown in excess of toxic metals in the soil (Tromp,

1989, Mazid *et al.*, 2010). In parallel, many environmental factors can influence the optimum conditions for plant growth and cell metabolism.

It may be resolved from the survey of literature cited above that 4-Cl-IAA and copper have a diverse role in physiological processes in plant. Therefore, the present study was designed to further explore the physiological role of 4-Cl-IAA in mungbean (*Vigna radiata* L. Wilczek) grown under copper stress.

Chapter - 3

Materials and Methods

MATERIAL AND METHODS

Proposed Study

To achieve the objectives, framed in chapter one, following studies were conducted to study the role of 4-chloroindole-3-acetic acid in *Vigna radiata* L. cv. T-44 treated with copper.

Seed

The seeds of *Vigna radiata* L. cv. T-44 were purchased from National Seed Corporation Ltd. Pusa, New Delhi, India. The healthy seeds were surface sterilized with 0.01% aqueous solution of mercuric chloride ($HgCl_2$) followed by repeated washing with double distilled water (DDW).

Hormone Preparation

4-chloroindole-3-acetic acid (4-Cl-IAA) was provided by Prof. K.C. Engvild, RISO national Laboratory, and Copenhagen, Denmark. A stock solution of 4-Cl-IAA was prepared by dissolving required quantity of the hormone in 5 cm^3 of ethanol in a 100 cm^3 volumetric flasks and final volume was made up to the mark by using DDW. The desired concentration of 4-Cl-IAA was prepared by the dilution of stock solution and Tween-20 (5%) was used as surfactant at the time of treatment .

Experiment

The surface sterilized of *Vigna radiata* (L) Wilczek var. T-44 seeds were soaked for 8 hours, in DDW (control) or 10^{-8} M aqueous solution of 4-Cl-IAA. These treated seeds were washed with DDW, to remove adhering solution and inoculated with specific *Rhizobium* sps. The experiment was conducted with 80 pots in such a way that each treatment had 10 pots

(replicates) and within each pots there were three plants, under complete randomized design technique.

The sowing was done in earthen pots (6 inch diameter) filled with sandy loam soil and farmyard manure (mixed in the ratio of 6:1). Copper was given in the form of copper sulphate ($CuSO_4$) at the rate of different concentration i.e. 50, 100 and 150 mg/kg of soil.

The plants were harvested at 30 days after sowing (DAS) to make the following observation:

Parameters studied:

1. Length of shoot and root plant^{-1}
2. Shoot and root fresh mass plant^{-1}
3. Shoot and root dry mass plant^{-1}
3. Nodule number plant^{-1}
4. Nodule fresh and dry mass plant^{-1}
5. Leaf area
6. Chlorophyll content (SPAD value) in leaves
7. Membrane stability index in leaves
8. Electrolyte leakage in leaves
9. Leaf water potential
10. Lipid peroxidation in leaves
11. Photosynthetic parameters
12. Maximum quantum yield of PS II (Fv/Fm)
13. Nitrate reductase (NR) activity in leaves
14. Carbonic anhydrase activity in leaves

15. Proline content in leaves

16. Peroxidase activity in leaves

17. Superoxide dismutase activity in leaves

18. Catalase activity in leaves

Determination of growth parameters

The following methods were adopted to assess the following growth parameters

Shoot and root length plant^{-1}

One plant from each replicate was taken and length of shoot and root was measured in cms scale.

Fresh and dry of shoot and root plant^{-1}

Fresh mass of the shoot and root per plant was determined with the help of electrical balance. The plant samples were kept in an oven run at 60°C for 72 h. After 72 h, the samples were weighed on electrical balance to ascertain their dry mass.

Number of nodules plant^{-1}

Whole plant was uprooted with the precaution that the roots may not be damaged. They were washed under the running tap water and the number of nodules per plant was counted.

Nodule fresh and dry mass plant^{-1}

The nodules from each plant were picked and weighed. The nodules were then transferred to petriplates for drying in an oven, run at 80°C. This dried

material was weighed and dry mass of nodules per plant was recorded, respectively.

Leaf area

It was determined by gravimetric method where the leaf area of randomly selected leaves from each treatment, was determined by tracing its outline on the graph sheet.

Chlorophyll content

The SPAD chlorophyll in the fresh leaf samples was measured by using Minolta (SPAD) chlorophyll meter (SPAD-502) (Konica Minolta Sensing Inc., Japan).

Membrane Stability Index (MSI)

MSI was estimated by taking 200 mg leaf material in 10 cm^3 of DDW in two sets. One set was heated at 40°C for 30 min in a water bath and the electrical conductivity C_1 was measured by a conductivity meter. Second set was boiled at 100°C on a boiling water bath for 10 min and its conductivity was also measured by conductivity meter as C_2. MSI was calculated using the formula described by Sairam (1994)

$$MSI = [1 - (C_1/C_2) \times 100]$$

Electrolyte leakage

The total inorganic ions leaked out in the leaves were measured by the method described by Sullivan and Ross (1979).

Twenty leaf discs were taken in a boiling tube containing 10 cm^3 of deionized water. The contents were heated at 45°C (EC_a) and 55°C (EC_b) for 30

min each in water bath and respective EC were measured by conductivity meter. Later the contents were boiled at 100°C for 10 min and the EC was again recorded as EC_c. The electrolyte leakage was calculated by using the formula:

$$\text{Electrolyte leakage (\%)} = \frac{EC_b - EC_a}{EC_c} \times 100$$

Leaf water potential

The leaf water potential was measured with the help of PSYPRO Water Potential System (Wescor Inc. USA).

Lipid peroxidation

Lipid peroxidation rates were estimated by measuring the malondilaldehyde equivalent according to Hodges *et al.* (1999). 0.5 g of leaf was homogenized in a mortar with 80% ethanol (Appendix 8.1). The homogenate was centrifuged at 3000 rpm for 10 min at 4°C. The pellet was extracted twice with same solvent. The supernatants were pooled and 1 cm^3 of this sample was taken in a test tube with an equal volume of the solution comprised of 20% trichloroacetic acid (Appendix 8.2). 0.01% butylated hydroxy toluene (Appendix 8.3) and 0.65% thiobarbutyric acid (Appendix 8.4). Sample was heated at 95°C for 25 min and cooled at room temperature. Absorbance of the samples was recorded at 440, 532 and 600 nm. Lipid peroxidation rates equivalent were calculated by using the formula given by Hodges *et al.* (1999) and expressed as (n mol malondialdehyde cm^{-1}).

1) $[(Abs_{532} + TBA) - (Abs_{600} + TBA)] - [(Abs_{532} - TBA) - (Abs_{600} - TBA)] = A$

2) $[(A_{440} + TBA) - (A_{600} + TBA)] \times 0.0571 = B$

3) $C = \frac{A - B}{157 \times 10^3} \times 10^6$ or $C = \frac{A - B}{157} \times 10^3$

4) $MDA = C \times 3$

Photosynthetic measurements

The photosynthetic parameters (photosynthetic rate, stomatal conductance, water use efficiency, internal CO_2 concentration, and transpiration rate) were measured by using portable photosynthesis system (LI-COR 6400, Lindon, USA). The measurements were made on uppermost fully expanded leaves of the main branch of plants between 11 to 12h under clear sun light.

Maximum quantum yield of PS II (FV/Fm)

The Fv/Fm parameter was measured on the upper surface of the leaf by using portable photosynthesis system (LI-COR 6400, Lincoln, NE, USA). All the measurements carried out at a photon flux density (PFD) of 1500 µmol m-2 s-1, with a constant airflow rate 500 □mol s-1. The sampled leaf was dark adapted for 30 minutes prior to measurement of Fv/Fm..

Nitrate reductase activity

The activity of nitrate reductase was measured following the method laid down by Jaworski (1971), in fresh leaf samples. The leaves were cut into small pieces (1 cm^2). 200 mg of these chopped leaves were weighed and transferred to plastic vials. To each vial, 2.5 cm^3 of phosphate buffer (pH 7.5) (Appendix

1.1) nd 0.5 cm^3 of potassium nitrate (Appendix 1.2) solution was added followed by the addition of 2.5 cm^3 of 5% isopropanol (Appendix 1.3). These vials were incubated in a BOD incubator for 2 h at 30±2°C in dark. 0.4 cm^3 of incubated mixture was taken in a test tube to which 0.3 cm^3 each of sulphanilamide (Appendix 1.4) solution and NED-HCl (Appendix 1.5) were added. The test tube was left for 20 min for maximum colour development. The mixture was diluted to 5 cm^3 with DDW. The absorbance was read at 540 nm on spectrophotometer. A blank was run simultaneously with each sample. Standard curve was plotted by using known graded concentrations of $NaNO_2$ (sodium nitrite) solution. The absorbance of each sample was compared with that of the calibration curve and nitrate reductase activity [n mole NO_2 g^{-1} (FM) s^{-1}] was expressed on fresh mass basis.

Carbonic anhydrase (CA) activity

The activity of carbonic anhydrase in the leaves was measured by the method described by Dwivedi and Randhava (1974). The fresh leaf samples were cut into small pieces at a temperature below 25°C. 200 mg of these pieces were weighed and transferred to petriplates. The leaf pieces were cut further into smaller pieces in 10 cm^3 of 0.2M cystein hydrochloride (Appendix 2.1) and left at 4°C for 20 min. The leaf pieces were blotted and transferred to a test tube containing 4 cm^3 of phosphate buffer of pH 6.8 (Appendix 2.2). To this test tube, 4 cm^3 of 0.2M sodium bicarbonate (Appendix 2.3) solution and 0.2 cm^3 of 0.002% bromothymol blue (Appendix 2.4) was added. The test tube was shaken gently and left at 4°C for 20 minutes. CO_2 liberated by the catalytic action of CA on $NaHCO_3$ was estimated by titrating the reaction mixture

against 0.01N HCl (Appendix 2.6) using methyl red (Appendix 2.5) as indicator. In each sample the quantity of HCl used to neutralize reaction was noted and difference was calculated. A blank consisting of all the above components of reaction mixture, except the leaf sample, was run simultaneously with each set of samples. The activity of the enzyme was calculated by putting the values in the formula.

$$CA = \frac{V \times 22 \times N}{W} \text{ [mol } (CO_2) \text{ kg}^{-1} \text{ (leaf F.M.) s}^{-1}\text{]}$$

V = difference in volume (cm^3 of HCl used in control and test sample during titration)

22 = equivalent weight of CO_2

N = Normality of HCl

W = Fresh mass of tissue used

Proline content

The proline content in fresh leaves was estimated following the procedure adopted by Bates *et al.* (1973). 0.5 g of fresh leaf sample was homogenized in a mortar with 5 cm^3 of 3% sulphosalicyclic acid (Appendix 3.1). The homogenate was filtered and collected in a test tube with two washings with 5 cm^3 of sulphosalicyclic acid. 2 cm^3 each of glacial acetic acid and acid ninhydrin (Appendix 3.2) was added to the above extract. This mixture was heated in boiling water bath for 1 h. The reaction was terminated by transferring the test tubes in an ice bath. 4 cm^3 of toluene was mixed to the reaction mixture with vigorous shaking for 20-30 s. The chromophore (toluene) layer was aspirated and warmed to room temperature. The absorbance of red

colour was read at 520 nm against a reagent blank. The amount of proline in the sample was calculated by using a standard curve prepared from pure proline (range 0.1 – 36 μ mol) and expressed on fresh mass basis.

$$\mu \text{ moles of proline } g^{-1} \text{ tissues} = \frac{\mu \text{ g proline } cm^{-3} \text{ x } cm^{-3} \text{ toluene}}{115.5} \text{ x } \frac{5}{\text{g (sample)}}$$

where 115.5 is the molecular mass of proline.

Estimation of peroxidase, superoxide dismutase and catalase

500 mg of leaf tissue was homogenized in 5 cm^3 of 50 mM phosphate buffer (pH 7.0) (Appendix 7.1) containing 1% polyvinylpyrrolidone. The homogenate was centrifuged at 15,000 rpm for 10 minutes at 5°C and the supernatant obtained was used as extract for SOD, POX and CAT.

Peroxidase (POX)

The activity of POX was assayed by adopting the method of (Chance and Maehly, 1956). 3.0 cm^3 of pyragallol phosphate buffer (Appendix 6.1) and 0.1 cm^3 of enzyme extract, 0.5 cm^3 of 1% H_2O_2 (Appendix 6.3) were mixed in a cuvette and change in absorbance at 20s interval for a period of 3 min was read at 420 nm on a spectrophotometer. The control set was prepared by using DDW instead of enzyme extract.

Superoxide dismutase (SOD)

The activity of SOD was measured by the method of Beauchamp and Fridovich (1971). 3.0 cm^3 of reaction mixture was made containing 1.0 cm^3 of 50 mM phosphate buffer (pH 7.0) (Appendix 7.1), 0.5 cm^3 of 13 mM methionine (Appendix 7.2), 0.5 cm^3 of 75 mM NBT (Appendix 7.3), 0.5 cm^3 of

2 mM riboflavin (Appendix 7.4), 0.5 cm^3 of 0.1 mM EDTA (Appendix 7.5) and 0.1 cm^3 of enzyme extract . Riboflavin was added in the last. The absorbance of the reaction mixture was read at 560 nm on a spectrophotometer.

Catalase (CAT)

The estimation of CAT was done by permagnate titration method (Chance and Maehly, 1956). For estimation of catalase 3.0 cm^3 of phosphate buffer (pH 6.8) (Appendix 5.1), 1.0 cm^3 of H_2O_2 (Appendix 5.2) and 1.0 cm^3 of enzyme extract were mixed and this mixture was incubated at 25°C for 1 min. After incubation 10 cm^3 of H_2SO_4 (Appendix 5.3) was added to the reaction mixture. The mixture was titrated against 0.1N potassium permagnate (Appendix 5.4) to estimate the residual H_2O_2 until a joint purple colour persists for at least 15 s. Similarly, a control set was maintained in which the enzyme activity was stopped by the addition of H_2SO_4 prior to the addition of enzyme extract.

Statistical analysis

The values for the parameters were subjected to statistical analysis, following the standard procedure described by Gomez and Gomez (1984). The ANOVA test was applied to assess the significance of the treatment, at 5% level of probability. Standard error of the replicates was also calculated.

Chapter - 4

Results

RESULTS

Shoot and root length

Plants that were raised from the soil amended with differential levels of copper possessed significantly lower values for both shoot and root length (Table 1 and 2). A linear decrease in their length was observed with the increased level of metal in the soil. The highest level of metal decreased the values of shoot and root length by 57.03% and 60.0%, over their control plant. However the treatment of 4-Cl-IAA alone or as follow-up significantly improved the length and also neutralized the damage caused by the metal.

Fresh and dry mass of shoot

It is evident from Table 1 that the fresh and dry mass of shoot decreased with the increased level of metal in the soil. However, the treatment of the plant with hormone not only significantly improved the fresh and dry mass of shoot but also nullified the damaging effect of metal completely at its lower level and partially at its higher level.

Fresh and dry mass of root

These parameters followed a pattern comparable to that of the fresh and dry mass of shoot (Table 2). Here again the highest level of Cu (i.e. 150 mg kg^{-1} soil) was found to be the most toxic and decreased the values of these parameter by 69.0% and 56.55% as compared to their control. The subsequent treatment of these stressed plants (150 mg kg^{-1} soil) with hormone improved their values and showed 18.53% and 11.11% improvement over their stressed plants.

Table 1. Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on shoot length (cm), shoot fresh and dry mass (g) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)

Treatment	Shoot length $plant^{-1}$	Shoot fresh mass $plant^{-1}$	Shoot dry weight $plant^{-1}$
Control	20.18±1.21	7.11±0.42	2.19±0.13
4-Cl-IAA (10^{-8} M)	27.27±1.63	11.03±0.38	3.90±0.26
Cu (50 mg/kg)	16.54±0.54	5.68±0.37	1.88±0.11
Cu (100 mg/kg)	13.30±0.94	3.98±0.26	1.40±0.16
Cu (150 mg/kg	8.67±0.67	2.63±0.20	1.04±0.06
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	25.51±1.69	8.34±0.62	2.59±0.20
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	18.36±1.43	5.53±0.48	1.76±0.18
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	10.8±1.26	3.35±0.40	1.23±0.13
LSD at 5%	2.25	1.04	0.29

Table 2. Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on root length (cm), root fresh and dry mass (g) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)

Treatment	Root length $plant^{-1}$	Root fresh mass $plant^{-1}$	Dry root mass $plant^{-1}$
Control	12.20±0.66	6.63±0.29	1.45±0.087
4-Cl-IAA (10^{-8} M)	15.90±1.03	11.53±0.35	2.72±0.10
Cu (50 mg/kg)	9.51±0.64	4.97±0.21	1.17±0.06
Cu (100 mg/kg)	7.32±0.56	3.51±0.29	0.89±0.56
Cu (150 mg/kg)	4.88±0.35	2.05±0.19	0.63±0.040
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	14.26±1.08	6.79±0.51	1.50±0.087
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	10.20±0.86	4.40±0.49	1.06±0.087
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	5.95±0.70	2.43±0.36	0.70±0.051
LSD at 5%	1.5	0.59	0.25

Nodules number, their fresh and dry mass

The values summarized in table 3 showed that number of nodules, their fresh and dry mass exhibited inverse relation with the increased level of metal in the soil. Out of the different metal treatments (50, 100 or 150 mg kg^{-1} soil) 150 mg kg^{-1} of Cu had maximum impact which decreased number, fresh and dry mass of nodule by 38%, 45% and 36.36%, over the control respectively. Furthermore, the application of the hormone on these stressed plant partially neutralized the ill effect of the metal at this level but completely in the plant that were fed with the lower level of metal.

Leaf area and SPAD value of chlorophyll

Plants grown in the soil amended with different level of metal possessed comparatively smaller leaves and lower SPAD value of chlorophyll values as compared to their control plants. This degree of damage caused by the metal increased with the increased level of metal in the soil. However, the subsequent treatment of the plant with 4-Cl-IAA alone possessed larger leaves and maximum SPAD value of chlorophyll content that was 35.48% and 35.09% higher over their non-treated control plants. Moreover, the ill effect generated by metal was also significantly improved by the follow up treatment of hormone.

Electrolyte leakage and lipid peroxidation

These two parameters showed completely different response to varied level of metal as compared to others (table 5). Plants raised from the soil amended with different level of metal possessed significantly higher values for these two parameters as compared to their control. A linear increase was

Table 3. Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on nodule number, nodule fresh and dry mass (g) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)

Treatment	Nodule number $plant^{-1}$	Nodule fresh mass $plant^{-1}$	Nodule dry weight $plant^{-1}$
Control	17.05±1.05	0.20±0.012	0.033±0.001
4-Cl-IAA (10^{-8} M)	20.11±1.00	0.24±0.017	0.041±0.003
Cu (50 mg/kg)	15.68±0.89	0.17±0.011	0.029±0.001
Cu (100 mg/kg)	13.12±0.52	0.15±0.007	0.026±0.002
Cu (150 mg/kg)	10.57±0.42	0.11±0.017	0.021±0.003
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	20.86±1.21	0.22±0.018	0.036±0.002
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	15.49±1.09	0.18±0.013	0.031±0.002
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	11.74±1.08	0.12±0.011	0.024±0.002
LSD at 5%	1.44	0.02	0.004

Table4. Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on leaf area (cm^2), SPAD chlorophyll and membrane stability index (%) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)

Treatment	Leaf area	SPAD chlorophyll	Membrane stability index
Control	23.11±1.21	39.12±0.58	52.00±1.56
4-Cl-IAA (10^{-8} M)	31.31±1.20	52.85±0.52	61.62±2.01
Cu (50 mg/kg)	20.64±0.95	32.42±0.54	48.15±1.5
Cu (100 mg/kg)	16.87±1.02	28.15±0.44	42.64±1.15
Cu (150 mg/kg)	13.40±0.87	24.33±0.36	36.40±0.98
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	25.00±1.17	44.25±0.66	56.05±1.83
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	19.75±1.12	36.60±0.61	46.90±1.74
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	15.00±1.47	30.16±0.56	38.20±1.50
LSD at 5%	3.11	2.75	4.05

Table 5. Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on electrolyte leakage (%), leaf water potential (Mpa) and lipid peroxidation (nmol TBARS g^{-1}FM) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)

Treatment	Electrolyte leakage	Leaf water potential	Lipid per oxidation
Control	7.89±0.61	-1.89±0.018	117±3.15
4-Cl-IAA (10^{-8} M)	6.90±0.53	-1.35±0.012	93±2.64
Cu (50 mg/kg)	8.89±0.47	-2.19±0.022	131±4.20
Cu (100 mg/kg)	9.23±0.31	-2.43±0.026	144±4.65
Cu (150 mg/kg)	10.41±0.25	-2.67±0.028	158±5.25
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	7.01±0.57	-1.50±0.017	114±3.03
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	7.84±0.19	-1.88±0.015	104±3.39
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	9.57±0.42	-2.30±0.018	154±3.63
LSD at 5%	0.63	0.08	7.26

observed with the increased level of metal in the soil. However, the follow up treatment of these stressed plants with 4-Cl-IAA caused a marked decrease in both these parameters.

Membrane stability index and leaf water potential

Data depicted in table 4 and 5 clearly showed that membrane stability index and leaf water potential were maximum in the plants that received 4-Cl-IAA alone that were 18.5% and 28.57% higher, over the control. However, a linear decrease in both the attributes was observed with the increased level of metal in the soil. The follow up treatment with 4-Cl-IAA completely nullified the ill effect generated by the metal at its lower level and partially at its higher level.

Photosynthetic attributes

Data presented in table 6 and 7 showed that all the photosynthetic attributes exhibited a decreasing trend with the increased level of metal in the soil. The supply of Cu (150 mg kg^{-1} of soil) decreased the values of P_N, gs, Ci, WUE, E and maximum quantum yield of PSII (Fv/Fm) by 60.0%, 55.0%, 57.0%, 51.22%, 35.0% and 40.0%, over their control plants, respectively. However, subsequent treatment with 4-Cl-IAA, increased the values of all these parameters over that of the control and also completely neutralized the toxic effect generated by lowest level of metal (50 mg kg^{-1}), but partially that of the highest level (150 mg kg^{-1}). Maximum values for these attributes were recorded from the plants that received hormone alone.

Table 6. Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on net photosynthetic rate P_N (μM CO_2 $m^{-2}s^{-1}$), stomatal conductance gs (mol $m^{-2}s^{-1}$), and internal CO_2 concentration (Ci, ppm) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)

Treatment	Net photosynthetic rate	Stomatal conductance	Internal CO_2 concentration
Control	15.05±0.22	0.040±0.005	270±2.70
4-Cl-IAA (10^{-8} M)	20.33±0.19	0.052±0.009	356±3.56
Cu (50 mg/kg)	10.23±0.12	0.028±0.001	186±3.15
Cu (100 mg/kg)	7.67±0.10	0.023±0.001	148±2.62
Cu (150 mg/kg)	6.02±0.08	0.018±0.005	116±2.02
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	16.44±0.08	0.043±0.002	269±4.42
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	11.65±0.15	0.033±0.002	204±4.24
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	8.84±0.15	0.023±0.005	145±3.87
LSD at 5%	1.20	0.007	10.32

Table 7. **Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on water use efficiency (WUE), transpiration rate (E), and maximum quantum yield of PS II (Fv/Fm) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)**

Treatment	Water use efficiency	Transpiration rate	Fv/Fm
Control	0.289±0.002	2.37±0.023	0.80±0.008
4-Cl-IAA (10^{-8} M)	0.398±0.003	3.17±0.029	0.96±0.009
Cu (50 mg/kg)	0.234±0.002	1.99±0.018	0.60±0.007
Cu (100 mg/kg)	0.173±0.002	1.73±0.017	0.54±0.006
Cu (150 mg/kg)	0.141±0.004	1.54±0.033	0.48±0.005
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	0.298±0.003	2.72±0.031	0.84±0.009
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	0.230±0.005	2.07±0.028	0.68±0.008
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	0.367±0.006	1.72±.022	0.55±0.007
LSD at 5%	0.011	0.11	0.03

Activities of nitrate reductase and carbonic anhydrase

The leaves of the plants that were raised from the soil amended with differential level of metal (i.e. 50, 100 or 150 mg kg^{-1} soil) possessed significantly lower activity for these enzymes (i.e. nitrate reductase and carbonic anhydrase). The activity decreased with the increased level of metal in the soil. Application of 4-Cl-IAA to the plants not only increased the activity of these enzymes but also overcome the toxic effects of the metal, completely at the lower level of metal and partially at the higher level.

Antioxidant enzymes and proline content

The data depicted in tables 9 showed that the activity of these enzymes (POX, CAT and SOD) and proline content increased on being supplemented with the metal and/or hormone this increase further advanced with the increased level of metal in the soil thus the maximum values of these parameters were recorded from the plants that were raised from the soil amended with highest level of metal (150 mg kg^{-1} soil) and received hormone as a follow-up treatment. It showed 111%, 72.0% and 68.3% higher POX, CAT and SOD activity respectively and 102% higher proline content over their control plants.

Table 8. **Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on nitrate reductase (NR) activity [n mole NO_2 g^{-1} (FM) s^{-1}], carbonic anhydrase (CA) activity [mol CO_2 kg^{-1}(F.M.) s^{-1}), and proline content (mg g^{-1}FM) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)**

Treatment	NR activity	CA activity	Proline
Control	407±12.21	1.54±0.04	9.03±0.27
4-Cl-IAA (10^{-8} M)	520±15.6	1.87±0.05	11.60±0.36
Cu (50 mg/kg)	317±9.99	1.30±0.04	12.19±0.34
Cu (100 mg/kg)	297±8.91	1.17±0.03	13.09±±0.39
Cu (150 mg/kg)	244±7.32	1.04±0.029	15.35±0.40
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	412±14.04	1.98±0.07	15.80±0.44
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	386±13.17	1.63±0.06	17.38±0.47
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	283±9.23	1.40±0.04	18.24±0.51
LSD at 5%	30.48	0.25	1.03

Table 9. **Effect of seed soaking (8 h) treatment of 4-Cl-indole-3-acetic acid (10^{-8} M) and/or copper (50, 100, 150 mg kg^{-1}) on peroxidase (POX) (unit g^{-1} (F.M)], superoxide dismutase (SOD) unit g^{-1} (F.M)] and catalase (CAT) (mM H_2O_2 decomposed g^{-1}(F.M)) in *Vigna radiata* (L.) Wilczek cv. T-44 at 30 DAS (Data are the mean of three independent replicates; ±= standard error)**

Treatment	Peroxidase	Superoxide dismutase	Catalase
Control	9.85±0.29	183±5.49	332±9.96
4-Cl-IAA (10^{-8} M)	12.67±0.46	205±6.84	388±13.05
Cu (50 mg/kg)	12.80±0.41	219±6.51	405±11.94
Cu (100 mg/kg)	14.03±0.48	253±7.11	441±12.50
Cu(150 mg/kg)	15.46±0.50	272±7.50	481±13.32
Cu (50 mg/kg) + 4-Cl-IAA (10^{-8} M)	15.25±0.54	253±7.95	501±13.74
Cu (100 mg/kg) + 4-Cl-IAA (10^{-8} M)	18.51±0.58	274±8.34	531±14.25
Cu (150 mg/kg) + 4-Cl-IAA (10^{-8} M)	20.83±0.65	308±8.73	571±15.00
LSD at 5%	1.30	11.64	21.30

Chapter - 5

Discussion

DISCUSSION

Among pollutants of agricultural soils, Cu has become increasingly hazardous due to its involvement in fungicides, fertilizers and pesticides. However, Cu at high concentration become strongly phytotoxic and cause inhibition of plant growth or even death in extreme cases. Moreover plants grown in the presence of high level of Cu normally show chlorotic symptoms and reduced biomass, this ultimately lead to the altered growth and development, and loss of crop productivity.

The reversible interconversion of CO_2 and HCO_3^- is catalyzed by carbonic anhydrase (CA), whose activity is largely determined by photon flux density, concentration of CO_2, the availability of Zn (Tiwari *et al.*, 2005) and the genetic expression (Kim *et al.*, 1994). However, in the present study, the stress generated by copper decreased the activity of CA (Table 8). This may be due to an inhibition and/or metabolic dysfunction of the enzyme protein under heavy metal stress (Hopkins, 1995), also the stress generated by heavy metal decreases the partial pressure of CO_2 in the stroma by inducing the stomatal closure (Barcelo and Poschenrieder, 1990). Fariduddin *et al.* (2009) also reported low CA activity in *Brassica juncea*, exposed to copper stress. However, the plants supplemented with 4- Cl-IAA, alone or in combination with copper elevated the activity of CA (Table 8) as auxin could have acted the specific genes to modify their state of expression because of their involvement in transcriptional and/or translational processes (Woodward and Bartel, 2005), hence increasing the level of required enzyme proteins. Moreover, higher CA activity in the presence of 4-Cl-IAA is supported by Ahmad *et al.* (2001b) who reported that auxin elevated the level of CA in *Brassica juncea*. The copper

stressed plants showed lower rate of net photosyntehsis that was accompanied by a significant decrease in stomatal conductance (gs), intercellular CO_2 (Ci,) water use efficiency and transpiration rate (E) along with maximum quantum yield of PS II (Fv/Fm) (Table 6 and 7). As heavy metal brought about the closure of stomata by decreasing the partial pressure of CO_2 in the stomata (Barcelo and Poschenrieder, 1990) this becomes marker cause of observed loss of Gs, Ci, and E. In addition to this, it was proposed that Cu interferes with the biosynthesis of the photosynthetic machinery by modifying the pigment and protein composition of thylakoid membranes (Lindon and Henriques, 1991; Maksymie *et al.*, 1994) that resulted a decrease in internal CO_2 concentration (Table 6). Decrease in CO_2 concentration may lead to excess reduction of PS I and depletion of $NADP^+$ pools that act as electron acceptor. The cumulative effect of all these altered process to a decrease in the net photosynthetic rate (Table 6). Beside this, the most apparent effect of Cu toxicity is on PS II which lead to inhibition of oxygen evolution accompanied by quenching of variable fluorescence (Samson *et al.*, 1988; Mohanty *et al.*, 1989). Moreover, Cu toxicity also affects the acceptor and donor rised of PS II (Yruela, 2005) that results in the loss of maximum quantum yield of PS II (Fv/Fm) (Table 7). Fariduddin *et al.* (2009) also reported the loss in the photosynthetic traits in *Brassica juncea* under different levels of copper. With the alteration in photosynthetic machinery, the presence of high concentration of Cu also significantly decreased the level of chlorophyll (SPAD value) (Table 4). The decrease in chlorophyll might be mediated through reduced uptake of Mg (Huang and Vitorello, 1996) that constitutes the integral part of the molecule. However, the plants raised from the seed soaked in 4-Cl-IAA exhibited the higher values of net photosynthetic rate and their related attributes (gs, Ci,

WUE and E) along with increased maximum quantum yield of PS II (Fv/Fm) (Table 6 and 7). Moreover, the use of 4-Cl-IAA also overcome the damaging effect of copper stress and increased the value for above said parameters in the plants exposed to different levels of copper (Table 6 and 7). The possible reason could be that Auxin acted at specific genes to modify their state of expression because of their involvement in transcriptional and/or translational process (Moore, 1989) hence increase in the level of required enzyme proteins. Moreover, this act of the auxin may also been the basic cause of auxin-induced enhancement of chlorophyll pigments and enhanced photosynthesis rate (Arteca and Dong, 1981) coupled with higher rate of phosphorylation (Chatterjii *et al.*, 1976) induced by Axuin which finally culminated into improved rate of photosynthesis and their related attributes (Table 6 and 7). Ali *et al.* (2007) reported that Auxin exhibited an elevated level of chlorophyll content and rate of photosynthesis in *Vigna radiata* which confirms our present finding. Moreover, Edward and Mohammad (1973), Okhi (1973) and Ahmad *et al.* (2001b) established a positive correlation between CA and photosynthesis which further strengthened the results.

In the present study, all the attributes (length of shoot and root, fresh and dry mass of shoot and root and leaf area) were significantly decreased in the plants that were exposed to different levels of copper (Table 1, 2 and 4). Although, Cu is known to be an essential oligonutrient for normal growth and development of plant but it becomes toxic even when the tissues contain slightly higher level of Cu than the optimal levels (Woolhouse, 1983; Fernandes and Henriques, 1991). In early stages of Cu toxicity, root growth was affected due to inhibition of cell elongation while in later stage of growth it was due to the reduced cell division (Barcelo and Poschenrieder, 2002;

Ciamporova, 2002) as well as Cu was shown to inhibit growth and also interfere with important cellular processes such as photosynthesis and respiration (Table 6) which lead to reduced biomass (Table 1 and 2). Moreover, Cu toxicity also interferes with water relations (leaf water potential) (Table 5) and membrane permeability (electrolyte leakage) (Table 5) (Detters *et al.*, 1986) and causes nutrient deficiencies mainly Fe, P, K, Ca and Mg (Haung and Vitorello, 1996). However, the seed soaked in 4-Cl-IAA and grown in the presence of Cu was found to be less toxic as compared to the seedling raised from water soaked control (Table 1, 2 and 5). Auxins are known to influence the cell division, cell elongation and/or cell differentiation (Sitbon and Perrot-Rechenmann, 1997). The earliest action is to alter the electrical properties of the plasma membrane (electrolyte leakage) (Table 5) by influencing the proton pump that activates the shift of protons into cell wall. In accordance with the "acid growth theory" of auxin action, modification of cell wall brings about its loosening (Cleland, 1999) and also has an impact on the functioning of the ionic channels thereby affecting the direction of the movements of ions and solutes and the turgor of the cells (Mcadonald, 1997). Moreover, auxins also induce expression of genes by altering the type, activity and the level of proteins (Sitbon and Perrot-Rechenmann, 1997). The cumulative effect of all these modified process improved the plant growth, also in the plants that were subjected to Cu stress. Mangus *et al.* (1997) reported that 4-Cl-IAA improved the fresh and dry mass of the *Pisum sativum* plants. The presence of heavy metal in the soil at a stress level is known to decline its microbial population (Rana and Ahmad, 2002). Therefore, a decrease in the nodule number, their fresh and dry mass was observed (Table 3). Thus the metal is said to be most toxic for nitrogen fixation, in various group of plants (Prasad, 1995). However,

the phytohormones are recognized to determine establishment, development and also the efficiency of the nodules (Dart, 1977 and Hopkins, 1995). Therefore, the exogenous application of auxins to alfa alfa (Gruodien and Zvizonaite, 1971) and groundnut (Srinivasan and Gopal, 1977) promoted nodulation in the former and nodule mass in the latter. The other hormones e.g. cytokinin (Nandwal and Bharti, 1982), gibberellins (Bishoi and Krishna, 1970) and brassinosteroids (Ali *et al.*, 2007) are also known to influence these processes in various plant types. This acquired efficiency in the plants, resulting from the seeds given pre-sowing seed treatment with auxin, had more nodule number, nodules fresh and dry mass (Table 3). Moreover, the auxin also neutralized the damaging effect of the metal to a limited extent (50 mg kg^{-1}). However, the possible regulatory role of auxin to normalize the above factors to make available more and more carbohydrates for speeding up the activity of bacteria.

Excess Cu in the soils leads to significant reduction in activity of nitrate reductase (NR) (Table 8). It may be due to the inhibition and/or metabolic dysfunction of the enzyme protein (Hopkins, 1995). Moreover, the metal also has an impact on the activity of plasma membrane bound protein (Obata *et* al., 1996) and the fluidity of the membrane (Meharg, 1993), restricting the uptake of nitrate (Harnandez *et al.*, 1996), the inducer of substrate of NR (Campbell, 1999). However, the application of 4-Cl-IAA alone or in combination with Cu elevated the activity of NR. The activity of NR is regulated by the substrate (NO_3^-), light, transcriptional state of the plant and metabolism/ signal transducers (Campbell, 1999). Moreover, the auxins also modify the activity of membrane bound ATPase and ionic channels, at the level of the roots, affecting the solute uptake and the cellular turgor

(Macdonald, 1997) which could have facilitated the influx of NO_3^- from the soil. It is well-documented that the auxin (4-Cl-IAA) increase the uptake of NO_3^- (Ahmad and Hayat, 1999) and also affects the transcription/translation process (Wood-ward and Bartel, 2005). Auxin have also been reported to stimulate the activity of NR in pea seedlings (Ahmad and Hayat, 1999), mustard plants (Ahmad *et al.*, 2001a) and germinating seeds of chickpea (Ali *et al.*, 2007a), pea and mungbean (Ahmad and Hayat, 1999, Ali 2006).

Membrane damage of plants could indirectly be evaluated by measuring solute leakage (electrolyte leakage) from cells (Ekmekci *et al.*, 2007) and membrane stability index (Ali *et al.*, 2008). Moreover, in the present study, the plant exposed to Cu sowed lesser membrane stability index (Table 4) and higher lipid peroxidation (Table 5). It well known fact that Cu toxicity interferes with water relation and membrane permeability, thus alters membrane stability index (Detters *et al.*, 1986) and transition metal like iron and copper catalyze the formation of hydroxy radicals from H_2O_2 and alkoxy or peroxyl radicals that result in oxidative stress (lipid peroxidation) in the cells (Halli well and Gutteridge, 1984; Schutzendubel and Polle, 2002). The cumulative effect of enhanced membrane stability index and reduced lipid peroxidation in the presence of auxin (Table 4 and 5) might have protected the plants against Cu-stress. The possible reason behind this may be that phytohormones generated such a response because of their involvement in the modification and/or manipulation of the plasma membrane structure/permeability under stress conditions (Khripach *et al.*, 1999; Bajguz, 2000).

In a natural course, the plants exposed to stress generated a larger quantity of reactive oxygen species (ROS) (Schutzendebell and Polle, 2002) that may oxidize proteins, lipids and nucleic acids, resulting in the abnormalities at the level of the cell (di Toppi and Gabbrielli, 1999). Although oxidative stress is well known to be induced by heavy metal directly and considering Cu is an efficient catalyst in formation of reactive oxygen species (ROS) (Yruela, 2005) therefore oxidative burst under Cu stress lead to elevate the antioxidant systems. However, the plants are capable to counter successfully such stress conditions by inducing the synthesis of antioxidant metabolites (ascorbate, glutathione, tocopherol and proline) and enzymes (superoxide dismutase, catalase, peroxides and glutathione reductance) that provide additional power of resistance to neutralize the toxic effects of the stress generated through ROS (Schutzendubel and Polle, 2002) such as superoxide radical, hydroxyl ions and H_2O_2. Moreover, the level of these enzymes in copper stressed plants increased further in the presence of auxin (Table 8 and 9).

The elevation in the activities of antioxidant enzyme by application of phytohormones might be due to *ATPAZ* and *ATP24a* genes coding peroxidases which were constitutively up-regulated in the *det2* Arabidopsis mutant (Goda *et al.*, 2002). The enhanced activities of antioxidant enzyme seems to be the result of *de-novo* synthesis and/or activation of the enzymes, mediated through transcription and/or translation of specific genes (Khripach *et al.*, 1999; Bajguz, 2000) that resulted in the addition of more strength to stressed plants to resist toxicity generated by Cu.

Conclusion

Critical assessments of the present investigation provide some important clues about the physiological role of 4-Cl-IAA in plants grown under Cu stress which is listed below:

1. Stress generated by lower concentration (50 mg/kg) of Cu interacted positively with either of the 4-Cl-IAA (10^{-8}M).
2. The level of antioxidant system (superoxide dismutase, catalase, peroxidase and proline) increased in response to Cu stress that further improved by 4-Cl-IAA treatment. Therefore, it may be suggested that the elevated level of antioxidant system at least in part, was responsible for the development of resistance against copper stress in *Vigna radiata*. The increase in the degree of resistance due to application of 4-Cl-IAA was reflected in improvement of plant growth, photosynthesis and related process, in the presence of copper.

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Appendix

APPENDIX

PREPARATION OF REAGENTS

1. Reagent for the estimation of nitrate reductase (NR) activity

1.1 0.1 M phosphate buffer (pH 7.5)

27.2 g of KH_2PO_4 and 45.63 g of $K_2HO_4.7H_2O$ were dissolved separately in 1000 cm^3 of DDW. The above solutions of KH_2PO_4 and $K_2HPO_4.7H_2O$ were mixed in the ratio of 16:84.

1.2 0.2 M KNO_3

20.2 g of KNO_3 was dissolved in sufficient DDW and final volume was made upto 1000 cm^3, using DDW.

1.3 5% Isopropanol

5 cm^3 of isopropanol was pipetted into sufficient DDW and final volume was made up 100 cm^3, using DDW.

1.4 1% Sulphoanilamide

1 g of sulphanilamide was dissolved in 100 cm^3 of 3N HCl. 3N HCl was prepared by dissolving 25.86 cm^3 of HCl in sufficient DDW and final volume was maintained to 100 cm^3, by using DDW.

1.5 0.02% N-1-nepthyl ethylene diamine dihydrochloride (NED-HCl)

20 mg of NED-HCl was dissolved in sufficient DDW and final volume was made upto 1000 cm^3, by using DDW.

2. Reagents for carbonic anhydrase (CA) activity

2.1 Cystein hydrochloride solution (0.2 M)

48 g cystein hydrochloride was dissolved in sufficient DDW and final volume was made upto the 1000 cm^3, by using DDW.

2.2 Sodium Phosphate buffer

27.8 g NaH_2PO_4 and 53.65 Na_2HPO_4 was dissolved each separately in sufficient DDW and final volume was made 1000 cm^3. 51 cm^3 of

NaH_2PO_4 and 49 cm^3 of Na_2HPO_4 were then mixed to get the required solution.

2.3 Alkaline sodium bicarbonate solution

16.8 g sodium bicarbonate ($NaHCO_3$) was dissolved in aqueous 0.2M NaOH solution [0.8 g NaOH (1000 cm^3)$^{-1}$] and final volume was made upto 1000 cm^3 with DDW.

2.4 0.002% Bromothymol blue

0.002 g of bromothymol blue was dissolved in sufficient DDW and the final volume was made up to 100 cm^3, by using DDW.

2.5 HCl (0.05 N)

4.30 cm^3 of pure HCl was pipetted in sufficient DDW and final volume was made up to 1000 cm^3, by using DDW.

2.6 Methyl red indicator

A pinch of methyl red was dissolved in sufficient ethanol and final volume made 100 cm^3 using ethanol.

3. Reagent for the estimation of proline

3.1 3% Sulphosalisylic acid

3 g of sulphosalicylic acid was dissolved in sufficient DDW and final volume was maintained 100 cm^3 by using DDW.

3.2 Acid ninhydrin solution

1.25 g of ninhydrin was dissolved in a mixture of warm, 30 cm^3 of glacial acetic acid and 6 M phosphoric acid (pH 1.0) with agitation till it got dissolved. It was stored at 4°C and used within 24 h.

The 6M phosphoric acid was prepared by mixing 11.8 cm^3 of phosphoric acid with 8.2 cm^3 of DDW.

4. Reagents for estimation of lipid peroxidation

4.1 80% Ethanol

80 cm^3 ethanol was mixed in 20 cm^3 of DDW.

4.2 20% Trichloroacetic acid

20 cm^3 of trichloroacetic acid was mixed in 80 cm^3 of DDW.

4.3 0.01% Butylated hydroxytoluene

0.01 cm^3 of BHT was pipetted into sufficient DDW and final volume was made up to 100 cm^3, using DDW.

4.4 0.065% Tribarbutyric acid

0.65 g of thiobarbutyric acid was dissolved in sufficient DDW and final volume was made up to 100 cm3, by using DDW.

5. Reagents for estimation of CAT activity

5.1 Phosphate buffer (0.1M) for pH 6.8

3.54 g of Na_2HPO_4 was dissolved in 100 cm^3 of DDW and 3.72 g of NaH_2PO_4 was added to 100 cm^3 of DDW. To this 12.3 cm^3 of Na_2HPO_4 was added to 87.7 cm^3 of NaH_2PO_4.

5.2 H_2O_2 (0.1 M)

0.34 cm^3 of H_2O_2 was added to 100 cm^3 of DDW.

5.3 Sulphuric acid (2%)

2 cm^3 of H_2SO_4 was added to 98 cm^3 of DDW.

5.4 0.1N potassium permanganate

This was made by dissolving 0.162 g of $KMnO_4$ in 500 cm^3 of DDW.

6. Reagent for the estimation of peroxidase activity

6.1 Pyragallol phosphate buffer

It was prepared by mixing 25 ml of pyragallol in 75 ml phosphate buffer (pH 6).

6.2 Phosphate buffer (pH 6)

3.54 g of Na_2HPO_4 was dissolved in 100 cm^3 of DDW and 3.72 g of NaH_2PO_4 was added to 100 cm^3 of DDW. To this 12.3 cm^3 of NaH_2PO_4 was added to 87.7 cm^3 of NaH_2PO_4.

6.3 1% H_2O_2

1 cm^3 of H_2O_2 was pipetted into sufficient DDW and final volume was made up to 100 cm^3 using distilled water.

7. Reagent for the estimation of superoxidase activity

7.1 Phosphate buffer (50 mM) for pH 7.0

It was prepared by mixing 1.78 g Na_2HPO_4 and 1.56 g of NaH_2PO_4 in 100 cm^3 of DDW separately and mixing 91.5 ml of Na_2HPO_4 with 8.5 ml of NaH_2PO_4.

7.2 Methionine (13 mM)

It was prepared by dissolving 0.193 g of methionine in 100 cm^3 of DDW.

7.3 Nitrobluetetrazolium (NBT) (75 mM)

6.13 mg of NBT was dissolved in 100 cm^3 of DDW.

7.4 Riboflavin (2M)

0.732 mg of riboflavin was dissolved in 100 cm^3 of DDW.

7.5 EDTA (0.1M)

2.92 g EDTA was dissolved in 100 cm^3 of DDW.